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ABSTRACT

Utilizing the lateral buds of known high rubber producing plants as explants in culture medium specifically designed to engender shoot development and to prevent callus formation, unlimited numbers of replicate plants can be produced. Each has the same genotype as the parent. This procedure has long been used to rid plants of virus, since the latter generally does not occur in the embryonic tissues of the bud; it also, by virtue of its axenic nature, eliminates all microorganisms characteristic of the parent plant. Auxins had been found essential to callus formation, but since the latter is known to bring about chromosomal aberrations, it was avoided. The cytokinin benzylaminopurine strongly stimulated shoot growth, and the number of regenerated buds on the inoculum was proportional to its concentration. These buds produced shoots several centimeters in length which were caused to root on medium containing indolebutyric acid. Transferred to the septic condition of soil, the plantlets were gradually brought into full sunlight where they showed a brief vegetative growth with production of mature type leaves, and flowered. In contrast, seedlings of the same age remained vegetative. Chromosome studies of root smears from the tissue cultured plantlets showed that $2n = 36$, the normal number for sexually reproducing guayules.

INTRODUCTION.-- The importance of maintaining the genotypes of plants that produce a high amount of rubber was early recognized in this work, and procedures were established for the cloning of such plants by tissue culture. The advantages of this technique are that unlimited numbers of plants of the original genotype can be produced from a single high-yielding individual. The axenic procedure of tissue culture necessarily rids the explant of all its surface-living microorganisms, and the use of the semi-microscopic shoot tip as inoculum is a well-known method of ridding higher plants of viruses. Since it is known that the precursory formation of callus in tissue culture cloning of flowering plants often leads to chromosomal aberrations, the present work utilized culture media that prevented the development of such undifferentiated tissue from which regeneration of buds occurred. Since auxin, both natural as well as synthetic, engenders formation of callus in plant tissue cultures, its use was avoided. In contrast, cytokinins, which are entirely synthetic, and consist of various chemical substitutions of such nucleic acid components as adenine, (e.g., kinetin or N⁶-furfuryl-adenine), stimulate the formation of buds and no callus is developed. Our preliminary work with experimentally formed callus tissue showed no microchemically detectable rubber. It was decided therefore to clone plants that were known, by biochemical tests, to be high producers of rubber. Since the guayule plant at maturity shows prolific formation of lateral buds, the majority of which can be removed without affecting the growth of the parent plant, they were excised as explants for tissue culture cloning. By variations in the culture medium, each such bud can be caused to develop into one to many (up to fifty) new plants. Since it is known that cytokinin inhibits formation of roots, it was necessary to place the regenerated shoots upon medium containing auxin in order to regenerate the former.

MATERIALS AND METHODS.-- The most regenerative explants from mature plants of Parthenium argentatum (#593 from the University of Arizona, Tucson) were small shoot tips. These explants were excised according to the procedure described by Ball (1946) and measured 1 x 1 x 1 mm. and contained the shoot apex, the three youngest foliar primordia and a small extent of basal pith parenchyma. The buds of mature plants prior to dissection were surface-sterilized by immersion in 3 per cent sodium hypochlorite, rinsed in 50 per cent ethanol and lastly in sterile distilled water. The inocula were excised by microscalpels made according to the technique of Ball (1946) and placed upon slants of agar culture medium consisting of the minerals of Murashige and Skoog (1962) and 2 per cent sucrose (W/V); the medium was solidified by 1.3 per cent agar. All constituents of the medium, except the agar, were filter-sterilized by being forced through a Millipore filter (pore size 0.4 micrometers). The inocula on the agar, in culture tubes 25 x 200 mm., were grown under light of 300 f.c. derived from an equal-wattage combination of white fluorescent tubes and 25-watt incandescent bulbs.

Auxins, e.g., indoleacetic acid, 1-naphthaleneacetic acid, at concentrations of 0.1 milligram per liter, were inhibitory to growth of the explants. Cytokinins, however, were stimulatory to growth by cell division. The most satisfactory cytokinin was N⁶-benzyladenine, or benzylaminopurine, BAP. On culture medium consisting of minerals, sugar and water, growth consisted of the limited development of foliar primordia already present on the inoculum, and there was no development of basal parenchyma. The leaves that showed limited maturation were without the lobes characteristic of the species, thus constituting the juvenile type. (Fig. 1).

RESULTS.-- The inocula responded quickly to the presence of BAP at 0.1 mg/l, produced additional leaves which became lobed in subsequent development, thus constituting the mature type. The basal parenchyma bore adventitious buds (Fig. 2).

Higher concentrations of the cytokinin accelerated these developments. At 1 mg/l new lobed foliar primordia were quickly produced, along with additional adventitious buds on the basal parenchyma (Fig. 3). At 2 mg/l the regenerative process was temporarily slowed (Fig. 4), but later showed the same development. When these cultures on higher concentrations of cytokinin were allowed to grow for about a month, they became masses of adventitious shoots on medium with 1 mg/l BAP (fig. 5), and mostly buds on medium with 2 mg/l BAP (Fig. 6). The latter high concentration of cytokinin inhibited shoot elongation.

After a month's growth upon the cytokinin medium, the cultures were either transferred intact, or as separate excised shoots, to rooting medium. The latter contained no cytokinin, but included 3 per cent B-indolybutyric acid. The numerous shoots from original growth upon medium containing 1 mg/l BAP were separated and their bases inserted individually into tubes of rooting medium where they produced adventitious roots from the basal parenchyma. (Fig. 7). Such plantlets were always short and thick-stemmed, and the basal parenchyma large. Plantlets produced upon medium containing 0.1 mg/l BAP were always slender and had small basal parenchyma (Fig. 8). Within thirty days most of the plantlets had produced numerous adventitious roots (Fig. 9).

Although these plants had developed in axenic conditions, transfer to non-sterile soil brought about few losses. Microorganisms of soil had no deleterious effect. The plantlets were transferred to plastic tubes of soil (Berkeley mix of peat, sand and loam) and protected for the first week in the greenhouse by an encompassing glass culture tube (40 mm dia.). They were subsequently conditioned gradually to higher light intensity in the lath house, transferred to pots and grown in full sunlight out of doors.

Along with the potted plantlets, equal-aged seedlings were grown in full sunlight for thirteen months. During the first six months a few of the plantlets

flowered, but none of the seedlings did so. At thirteen months, all (30 of 30) plantlets flowered (Fig. 10, 11), but only one (1 of 20) of the seedlings did so. Most of the latter had continued vegetative growth, with no signs of inflorescence stalks (Fig. 12). The morphology of the flowers in the inflorescences on the plantlets was normal (Fig. 13).

The chromosome counts of the plantlets and of the seedlings were $2n = 36$. This chromosome number, according to the NAS report (1977) is the normal one for sexually reproducing plants of guayule. The finding of the normal number of chromosomes in the tissue culture derived plantlets is evidence that the procedures utilized in their derivation had not induced abnormal cytology.

DISCUSSION.— A tissue culture method has been described that successfully clones an individual plant of Parthenium argentatum. This procedure can now be used to make an indefinite number of replicates of a high-rubber yielding plant for commercial purposes or for breeding. Depending upon the concentration of the cytokinin, e.g., benzylaminopurine, a single explant can be caused to produce either a few shoots, or up to fifty. Each shoot can then be rooted, transplanted to soil where it will grow into a mature rubber-yielding plant.

With the successful achievement of this in vitro propagation procedure, it is now possible to institute an improvement program for rubber production by Parthenium argentatum. It will be important, both practically and theoretically, to ascertain whether plants derived by tissue culture under the influence of a cytokinin, will carry on the biochemical capacity for rubber production of and original plant that contributed the explants. While the present work demonstrated that there was no change in the chromosome number in plantlets produced under the influence of a cytokinin, it will be necessary to test the biochemical competence of resulting mature plants.

The donor plants were mature and bore numerous inflorescences. This mature state was perpetuated in the plantlets produced by tissue culture, for most of

them flowered during their growth in soil by thirteen months. Exceptional ones that remained vegetative had been previously defoliated by insects. In contrast, seedlings of the same age rarely flowered. This perpetuation of the mature state in the tissue cultured plantlets will be an important time-saver for breeding.

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- Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15: 473-498.
- National Academy of Sciences, Washington, D.C. Guayule, an alternative source of natural rubber. Washington, D. C., Library of Congress Catalog No. 72-62525. ix + 79 pp.



Figure 1. Explant grown 23 days without cytokinin. Leaves are unlobed, and no basal parenchyma occurred. No further development was noted after prolonged culture. 6X.



Figure 2. Explant grown 14 days on medium containing 0.1 mg/l BAP. New lobed leaves were produced and the basal parenchyma bore new adventitious buds. 6X.



Figure 3. Inoculum grown on medium containing 1 mg/l BAP for 14 days. In this brief interval new lobed leaves had been produced, and adventitious buds formed upon the basal parenchyma. 6X.



Figure 4. Inoculum grown on medium containing 2 mg/l BAP for 14 days. A slight inhibition due to the high concentration of cytokinin slowed development which later resumed. 6X.



Figure 5. Growth of an inoculum for one month on medium containing 1 mg/l BAP. So many adventitious shoots were produced that the culture had become a mass of them. Most buds showed development of elongated stems. 6X.



Figure 6. Growth of an inoculum for one month on medium containing 2 mg/l BAP. Numerous adventitious buds arose, but their stems usually elongated only slightly, apparently due to the high concentration of cytokinin. 6X.

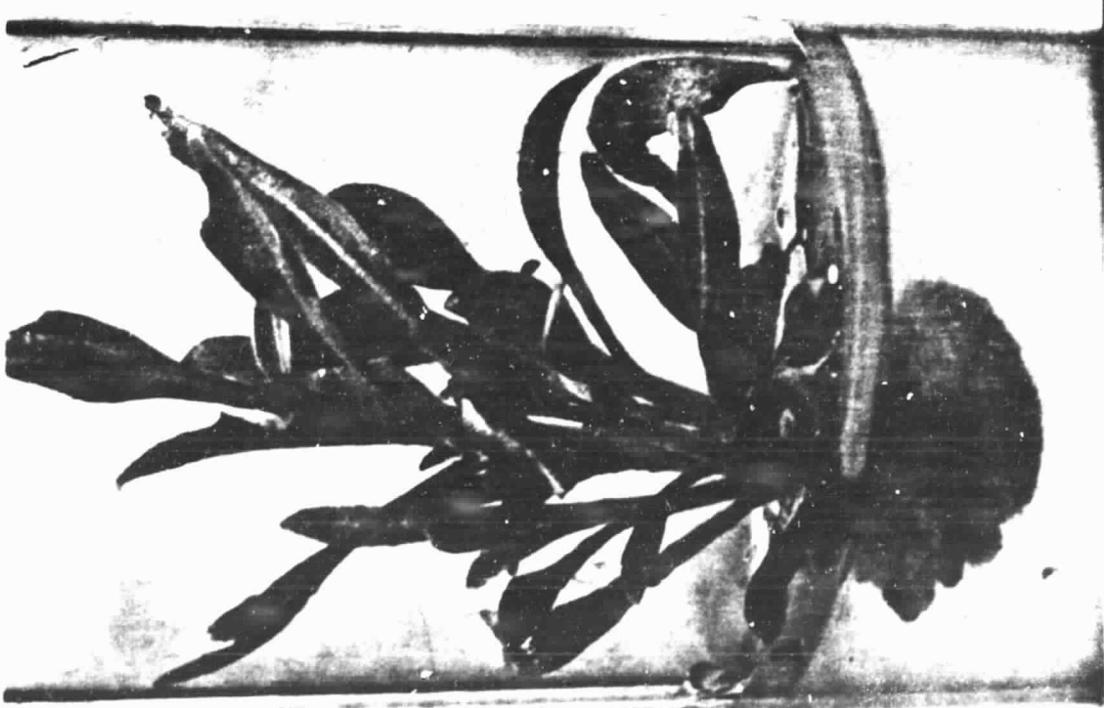


Figure 7. Shoot grown 30 days on medium with 1 mg/l BAP, then 28 days on rooting medium. Shoot is short and thick, the basal parenchyma large, with beginning adventitious roots. 4X.



Figure 8. Shoot grown 30 days on medium with 0.1 mg/l BAP, then 28 days on rooting medium. Shoot is elongate and slender, the basal parenchyma small. 4X.

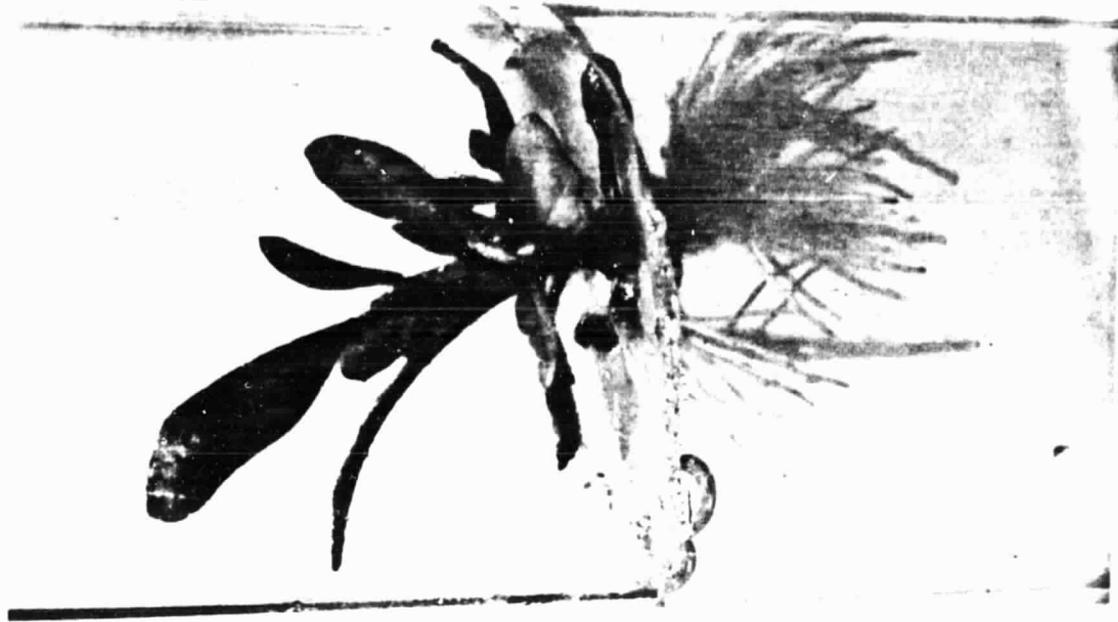


Figure 9. Shoot grown 30 days on medium with 1 mg/l BAP, then 28 days on rooting medium. Shoot is short and thick, and from the basal parenchyma numerous adventitious roots have arisen and grown into the agar. 4X.

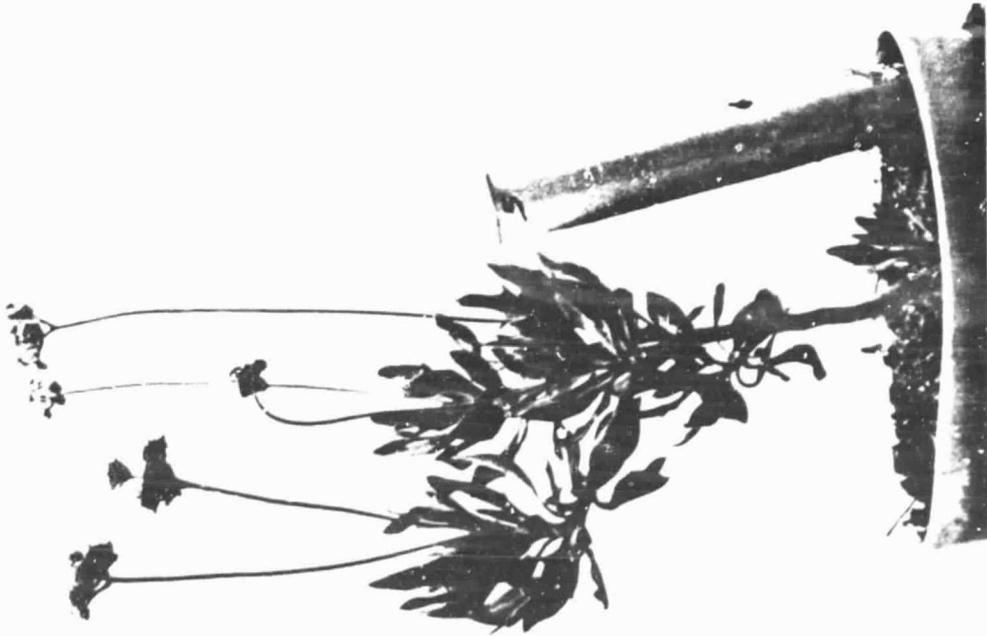


Figure 10. Plantlet in soil 13 months that bore five inflorescence stalks, each bearing multiple heads. 1/3 X.

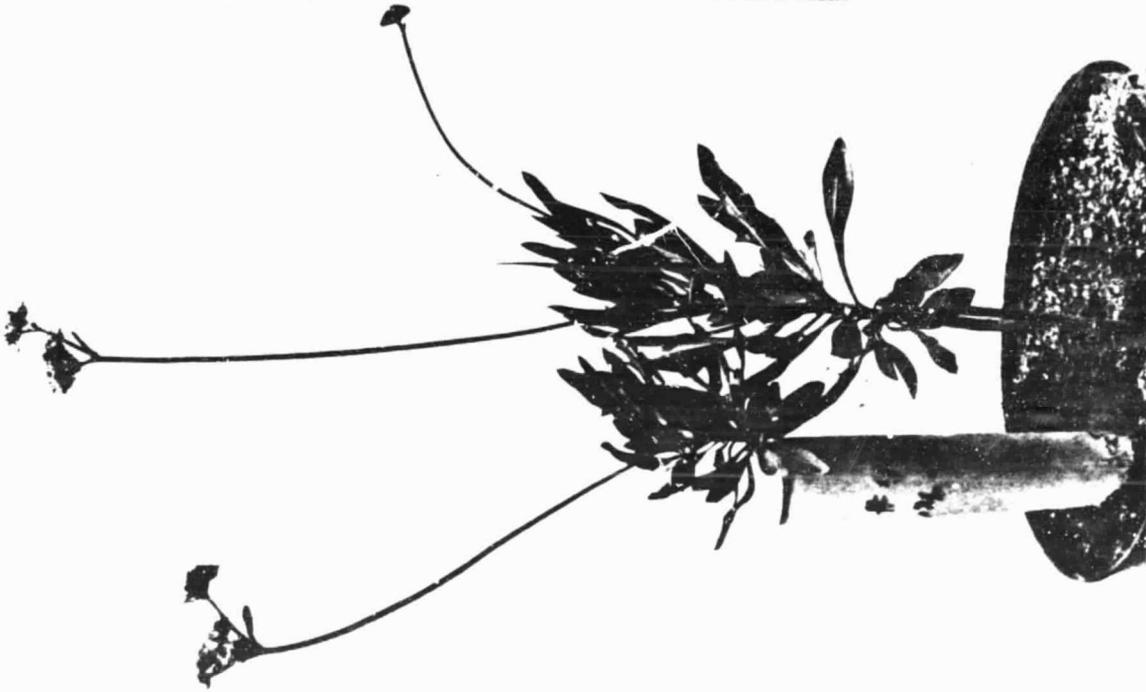


Figure 11. Plantlet in soil 13 months that bore three inflorescence stalks, each bearing multiple heads. These "extra" stalks derived from the persistence and sprouting of adventitious buds. 1/3 X.

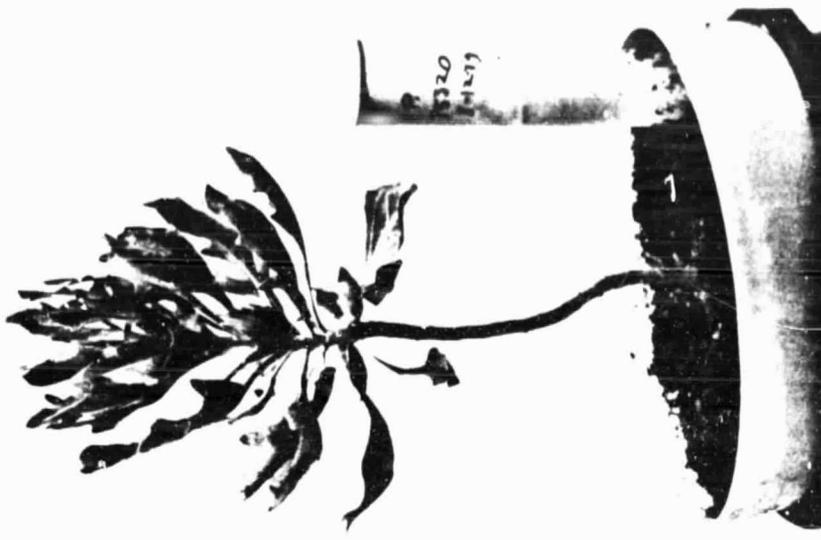


Figure 12. Seedling grown in pot of soil for 13 months with no sign of development of inflorescence. 1/3 X.



Figure 13. Surface view of a triple head of flowers on an inflorescence stalk of a plantlet grown 13 months in soil in a pot. Both ray and disc flowers are shown, and the morphology of each is normal. 8X.